Turning into frogs: asymmetry in forelimb emergence and escape direction in metamorphosing anurans

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Luigi Zechini[1], Alison Lilley[2], Emily Waddell[2], Thomas J. Burns[2], J. Roger Downie[2] and Patrick T. Walsh[1*]

Running head: Lateralised forelimb emergence and turning

[1] Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3JT

E-mail: luigizechini@gmail.com

* Corresponding author: E-mail: Patrick.Walsh@ed.ac.uk; Telephone: 0131 650-5474

[2] School of Life Sciences, College of Medical, Veterinary & Life Sciences, Graham Kerr Building, University of Glasgow, Glasgow G12 8QQ.

E-mail: alisoncatherinelilley@gmail.com

E-mail: emilyhelen22@hotmail.com

E-mail: tomb-09@hotmail.co.uk

E-mail: Roger.Downie@glasgow.ac.uk

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ABSTRACT

There is considerable debate about the pattern and origin of laterality in forelimb emergence and turning behaviour within amphibians, with the latter being poorly investigated in tadpoles around metamorphic climax. Using six species of metamorphosing anurans, we investigated the effect of asymmetrical spiracle location, and disturbance at the time of forelimb emergence, on the pattern of forelimb emergence. Turning behaviour was observed to assess whether motor lateralisation occurred in non-neobatrachian anurans and was linked to patterns of forelimb emergence. Biases in forelimb emergence differed among species, supporting the hypothesis that asymmetrical spiracle position results in the same asymmetry in forelimb emergence. However, this pattern only occurred when individuals were undisturbed. Therefore, context at the time of the emergence of the forelimbs may be important, and might explain some discrepancies in the literature. Turning biases, unconnected to forelimb emergence, were found in Pipidae and Bombinatoridae, confirming the basal origin of lateralised behaviour among anurans. Turning direction in our metamorphs differed from the left-ward bias commonly observed in tadpoles, but may be analogous to the prevalent right-"handedness" among adult anurans. Therefore, the transitions occurring during metamorphosis may affect lateralised behaviour and metamorphosis may be fruitful for understanding the development of lateralisation.

Key words: handedness, laterality, forelimb emergence, turning, metamorphic climax

Introduction

Behavioural lateralisation, or the favouring of one side of the body in a bilateral organism (so called "handedness"), was once thought to be unique to humans and linked to the development of quintessential human traits (e.g. language: Broca, 1865). However, laterality and morphological asymmetries are now recognised in many non-human animals, including arthropods, fish, amphibians, birds, and mammals (Bradshaw & Rogers, 1993; Bisazza *et al.*, 1998; Vallortigara & Rogers, 2005; Vallortigara *et al.*, 2011; Ströckens *et al.*, 2013; Versace & Vallortigara, 2015). While there is growing evidence that both lateralised behaviours and some morphological asymmetries may be beneficial (Rogers *et al.*, 2004; Walsh *et al.*, 2011; Blackiston & Levin, 2013), the developmental and evolutionary origins of these lateral differences and what, if anything, links physical asymmetries with behavioural lateralisation are still poorly understood (Versace & Vallortigara, 2015).

Amphibians, particularly anurans, have emerged as a key group in which investigations of lateralised behaviour and morphological asymmetries are being conducted (reviewed in Rogers, 2002; Wassersug & Yamashita, 2002; Malashichev & Wassersug, 2004). These investigations have largely, but not exclusively, focused on three aspects: the lateralised behaviour of turning preference in anuran larvae (Oseen *et al.*, 2001; Wassersug & Yamashita, 2002); the asymmetrical emergence of the forelimbs at metamorphosis (Malashichev & Nikitina, 2002; Malashichev, 2002; Zechini *et al.*, 2015); and forelimb preference in juveniles and adults (reviewed in Ströckens et al., 2013).

Anuran amphibian forelimbs develop within the opercular cavity (or, in pipids, in separate brachial sacs) and, once well developed, emerge asymmetrically (Malashichev, 2002), through openings in the overlying tissue. The mechanisms causing the openings are: 1) rising thyroid hormone levels, leading to thinning and degeneration of the overlying tissues; and/or 2)

mechanical pressure from the underlying limb (Braus, 1906; Helff, 1926; Helff, 1939; Newth, 1949). Species level patterns in the order of forelimb emergence are often observed (e.g. left-bias in several ranids: Speidel, 1925; Helff, 1926; Malashichev, 2002; right-bias in *Bufo bufo*: Malashichev, 2002; or no bias in *Bombina bombina*: Malashichev, 2002) and have been linked to the position and numbers of spiracles. In tadpoles, spiracles can vary from a single midline or lateral (sinistral) spiracle to paired lateral spiracles, with forelimb emergence biases occurring where a single lateral spiracle leads to the forelimb on that side emerging first (Speidel, 1925; Borkhvardt & Ivanhintsova, 1994; Borkhvardt & Malashichev, 1997). However, there are inconsistencies among studies, even within the same species (e.g. *Rana pipiens*: left bias Helff, 1926; Dickerson, 1969; right bias Rugh, 1977).

Similarly, turning behaviour in tadpoles has been demonstrated to often have a distinct "handedness", with tadpoles predominantly making left turns particularly when startled (Wassersug & Yamashita, 2002). However, this is not consistent across, nor within, species (Yamashita et al., 2000; Oseen et al., 2001; Rogers, 2002). Most incidences of species level bias have been observed in neobatrachian species (e.g. ranids, bufonids and hylids), with those that diverged earlier in the anuran lineage (e.g. bombinatorids and pipids: Frost et al., 2006) possibly not exhibiting a preference in turning direction (Yamashita et al., 2000; Oseen et al., 2001). Furthermore, across species, the apparent left turning bias, if present, appears to diminish as tadpoles develop, with the strength of the left bias strongest in early stage tadpoles (Wassersug & Yamashita, 2002). However, the number of well-developed tadpole species (post-Gosner (1960) stage 39) investigated has so far been limited. The apparent leftward tadpole bias differs from a prevalent right forelimb preference in adult anuran amphibians (Rogers, 2002; Ströckens et al., 2013).

In this study we investigated the directional bias in forelimb emergence and turning behaviour in a taxonomically diverse set of larval anuran amphibian species at late developmental stages. The results provide insight into the current ambiguity surrounding the potential link between morphological and behavioural lateralisation in the emergence of forelimbs and turning behaviour in late stage larval anurans.

Method

The study species (Table 1), rearing conditions and experimental procedures were the same as presented in Zechini *et al.* (2015). To summarise the procedures in brief: all species, except *Bombina orientalis* (acquired at Gosner stage 37) were acquired as eggs from up to two pairings (Table 1), and then reared in the laboratory at varying densities in aerated, dechlorinated copper-free water at 21 ± 3 °C, on a 12:12 L:D photoperiod, and fed *ad libitum* daily. When individuals reached the stage prior to the onset of metamorphic climax (Walsh, 2010), where forelimbs were noticeable under the skin but had not yet emerged (Gosner (1960) stage 41; Nieuwkoop & Faber (1994) (NF) stage 57), they were isolated for inclusion in the study.

As part of a larger study, some individuals were separated to observe the duration between the emergence of the forelimbs, and others were used to test turning direction and the locomotory impacts of asymmetric forelimb emergence. Therefore, individuals, for inclusion in this study, were subjected to two treatments: one where they were left undisturbed prior to the emergence of the forelimbs; and the other where they were subjected to incidences of startling prior to either forelimb emerging.

Assessing the lateral bias in turning behaviour was performed in a swimming arena, 30 cm in diameter and underlain with 1 cm grid paper, using a FASTCAM-PCI high-speed camera (filmed at 250 frames per second) to capture footage of the tadpole's escape response after being

startled. Startling for each recording was done by a consistent discharge of air from a 1 ml Gilson pipette to the rear of the animal (Van Buskirk & McCollum, 2000) using the grid paper to line up the tadpole and the pipette. Each individual was recorded five times, with a 1 minute interval between each recording. Turning direction was assessed as the direction that the body was flexed during a c-start, which generally occurred 30 ms after startling. Turning bias was assessed in the same individuals: 1) just prior to either forelimb emerging (Gosner stage 41; NF stage 57); 2) after one forelimb had emerged; and 3) with both forelimbs emerged (Gosner stage 42; NF stage 58). The final assessment was performed as soon as both forelimbs emerged (within 12 hours), so that all observations on an individual were done within a few days and were conducted prior to tail re-absorption. Twenty-five *R. temporaria*, 25 *B. bufo*, 16 *X. laevis* and 30 *B. orientalis* were assessed for turning bias. All individuals experienced disturbance, resulting from repeated movement to and from the testing arena and the assessment of turning bias itself, at the time when the forelimbs were emerging.

For those observed for the duration between the emergence of the forelimbs (Zechini *et al.*, 2015), 17 *R. temporaria*, 20 *B. bufo*, 20 *X. laevis*, 49 *X. borealis*, 39 *X. tropicalis* and 22 *B. orientalis* tadpoles were used. All individuals found prior to either forelimb emerging were held under the same conditions, and the first forelimb to emerge was recorded, while only a subsample of these were filmed and reported in Zechini *et al.* (2015) due to limited numbers of cameras. Unlike individuals assessed for turning bias, all of these individuals were in isolated conditions and not disturbed, even for feeding, until both forelimbs emerged and they were removed from the study.

Chi-squared tests were used to determine whether each species had a bias in which forelimb emerged first, while binary logistic regression was used to analyse whether the first forelimb to emerge differed between the undisturbed and startled tadpoles. To determine whether

142 species were biased in their direction of turning when startled, each species at zero, one and both
143 forelimbs emerged, was analysed separately using Repeated G-tests for goodness of fit. Due to
144 the high frequency of heterogeneity, species level biases were confirmed using a modified
145 version of the laterality index for each individual as described by Bisazza et al. (2000), so that:

146 $$\text{Laterality Index} = \left(\frac{\text{Turns to the right} - \text{Turns to the left}}{\text{Turns to the right} + \text{Turns to the left}}\right)$$

147 A general linear mixed model (GLMM) was used to examine the Laterality Index scores, with
148 species and the number of forelimbs emerged as fixed factors, ID as a random effect and the first
149 forelimb to emerge (either the left or right) as a covariate. Only significant interactions were
150 retained in the model. Bias in the laterality index was analysed using one-sample t-tests, for each
151 species and at each stage of forelimb emergence.
152
153 **Results**
154 *Forelimb emergence bias*
155 Of the six species in our study, four (*Xenopus laevis*, *X. borealis*, X. *tropicalis* and *Bombina*
156 *orientalis*) did not differ in which forelimb emerged first, regardless of whether they were startled
157 prior to forelimb emergence or not (Table 2). *Xenopus laevis* and *B. orientalis* had individuals
158 subjected to both treatments, and there was no difference in which forelimb emerged first
159 between the two observational groups (*X. laevis*: Wald = 2.74, df = 1, p = 0.10; *B. orientalis*:
160 Wald = 2.30, df = 1, p = 0.13).
161 In both *R. temporaria* and *B. bufo*, the left forelimb emerged first more frequently when
162 they were observed for the duration between forelimb emergences, but not when they were
163 assessed for turning direction (Table 2). In *R. temporaria* (Wald = 4.65, df = 1, p = 0.031), but

not *B. bufo* (Wald = 2.76, df = 1, p = 0.10), there was a significant difference in forelimb emergence bias between the two observational groups (Table 2).

Tadpole turning bias

Species differed in their laterality index scores ($F_{3,274.62} = 4.19$, $p = 0.006$; Table 3). Laterality index scores were closest to 0 (no bias) when both forelimbs had emerged, compared to either no or one forelimb emerged ($F_{2,199.64} = 8.50$, $p < 0.001$). However, the forelimb to emerge first did not affect the laterality index score ($F_{1,274.62} = 0.09$, $p = 0.76$).

Bufo bufo was the only species assessed for the direction of turning that did not show a directional bias at any stage in the progression from neither to both forelimbs emerged (Table 3), and individuals were homogeneous in not showing a directional bias (Table 3). In contrast, *Xenopus laevis* exhibited a significant right bias in turning at each stage (Table 3). However, there was greater heterogeneity at no and one forelimb emerged (Table 3), with some individuals showing a very strong right bias while in others the right bias was less strong or individuals had a left bias.

Rana temporaria exhibited a significant right bias only at stage 41, when no forelimbs had emerged (Table 3), but there was a difference among individuals in the strength of their rightward bias (Table 3). When *R. temporaria* had one or both forelimbs exposed, they did not show a significant bias in either direction (Table 3). However, when one forelimb was exposed, there was significant heterogeneity indicating that some individuals did show a significant directional bias, but overall most individuals did not.

Bombina orientalis showed a consistent right bias when neither and one of the forelimbs had emerged (Table 3), but there was significant variation in the strength of the bias when one forelimb was emerged. After both forelimbs had emerged, the right bias diminished (Table 3).

Discussion

Forelimb emergence bias

We observed in all three pipid species and *B. orientalis* that although there was always asymmetry in forelimb emergence, with variable timing between the emergence of the first and second limb, there was no bias in which forelimb emerged first. This provides confirmation of the results of Borkhvardt & Malashichev (1997) and Malashichev (2002), and expands them to include two new species (*X. borealis* and *X. tropicalis*). We also observed that both *R. temporaria* and *B. bufo*, when not assessed for turning direction, demonstrated a left bias in forelimb emergence. These patterns of forelimb emergence bias provide support for the hypothesis that forelimb emergence and spiracle position are linked with limbs able to emerge more readily through a spiracle, which was proposed by Speidel (1925) and Borkhvardt & Malashichev (1997), but later contested by Malashichev (2002) due to observations of strong right bias in *B. bufo*. Any asymmetry in spiracle position, as in *Bufo* and *Rana*, may therefore lead to asymmetrical forelimb emergence showing the same directional bias, whereas symmetrical spiracles (either a single nearly midline, as in *Bombina*, or paired lateral spiracles as in *Xenopus*) do not lead to a consistent bias in which forelimb emerges first.

Unexpectedly, *R. temporaria* and *B. bufo* did not show any lateral bias in forelimb emergence when individuals were subjected to repeated startle stimuli during testing for turning direction. This result suggests that under different circumstances additional factors may drive forelimb emergence. When tadpoles approaching metamorphic climax are startled they often erratically move their forelimbs within the opercular chamber (PTW, personal observations). Therefore mechanical pressure from the elbows, which may be equal on both sides, may drive emergence. Conversely, without the repeated disturbance caused by the assessment of turning

behaviour at the time the forelimbs were about to emerge, the perforations that form with the impending onset of metamorphic climax might occur more readily on the side with the spiracle (Speidel, 1925), leading to the biases we observed. That circumstances occurring around the time of forelimb emergence play a role in the subsequent asymmetry (Versace & Vallortigara, 2015) may also explain the often contradictory, or at least variable results that have been reported on this phenomenon (Malashichev, 2002).

That there was such a stark difference in the lateral bias of forelimb emergence in *B. bufo* between our current study and Malashichev (2002), is surprising. Unfortunately, the conditions under which forelimb emergence occurred in Malashichev (2002) are not reported, so may have contributed. Alternatively, given the challenges of classifying the *Bufo bufo* species group (e.g. Garcia-Porta *et al.*, 2012; Arntzen *et al.*, 2013) there may be population differences in forelimb emergence asymmetry that supercedes associations with spiracle placement, which would warrant further investigation. However, with the exception of *B. bufo* where there is some ambiguity, the species in our current study and those cited within Malashichev (2002) (Bhati, 1961; Borkhvardt & Ivanhintsova, 1994) all conform to the hypothesized association between spiracle position and forelimb emergence. This suggests that the hypothesis may be more robust than previously considered. Ultimately, confirmation would require reconciling currently anomalous species (e.g. *Bufo bufo*) with this hypothesis or other hypotheses (e.g. link between alternate limb locomotion to lateralisations (Malashichev, 2006)), or determine the significance of context-dependent impacts on lateralisations.

Tadpole turning bias

All four species at all three stages, with the exception of *R. temporaria* and *B. bufo* with both forelimbs emerged, demonstrated a weak trend towards turning right when startled, while

236 forelimb emergences were either left-biased or no bias present. Furthermore, across all species,
237 the direction of turning was not affected by whether the right or left forelimb emerged first.
238 Surprisingly, *X. laevis* and *B. orientalis*, which did not exhibit a lateral bias in the emergence of
239 their forelimbs, showed the most persistent lateralised turning response. The literature on turning
240 bias is highly equivocal, often due to different methods being used across studies (Wassersug &
241 Yamashita, 2002), but our result was unexpected for two reasons. Firstly, the most commonly
242 observed turning bias, if present, appears to be a left bias in tadpoles (Wassersug & Yamashita,
243 2002; Rogers, 2002; Malashichev & Wassersug, 2004). Secondly, species of Pipidae and
244 Bombinatoridae have previously been shown to lack any lateral bias, either in tadpole turning
245 (Wassersug *et al.*, 1999; Goree & Wassersug, 2001) or in adult forelimb use (*B. orientalis*: Goree
246 & Wassersug, 2001; *B. bombina*: Malashichev & Nikitina, 2002) or turning (*Xenopus laeivs*:
247 Kostylev & Malashichev, 2007).
248 With respect to the right biased turning behaviour we observed, there is a possible
249 explanatory difference between the current study and previous work. Our study was specifically
250 focussed on late stage tadpoles just before and at the start of metamorphic climax. Most (8 out of
251 11) of the studies presented in Wassersug & Yamashita (2002) reporting a left bias did not
252 include individuals beyond Gosner stage 39. It has previously been observed that the prevalence
253 of the left bias diminishes as tadpoles develop (Wassersug *et al.*, 1999; Oseen *et al.*, 2001;
254 Malashichev & Wassersug, 2004). However, with the inclusion of our findings on late stage
255 tadpoles, the declining left bias may represent a transition from left bias to right bias with
256 development (Figure 1; Wassersug & Yamashita, 2002). This would be supported by the
257 prevalence of right limb bias, where biases occur, in adult anurans (Rogers, 2002, for exceptions
258 see *Bufo viridis*: Robins *et al.*, 1998). This could be due to the changes that occur in the transition
259 from tadpole tail driven locomotion to the inclusion of limbs in their locomotion, or neurological

changes in asymmetries observed during metamorphosis (Proshchina & Savel'ev, 1998). However, given that the right bias diminished as one or both forelimbs emerged in two of the four species that were assessed, this is not conclusive. Ultimately, greater focus on the developmental progression of lateral bias across all stages is required.

Our results are the first to show a lateral turning bias in a pipid or a bombinatorid, both sister groups to the neobatrachians (Frost *et al.*, 2006). This indicates that the origin of this phenomenon in anurans is more ancient than previously suspected (Wassersug *et al.*, 1999; Goree & Wassersug, 2001; Briggs-Gonzalez & Gonzalez, 2016) and conforms with lateralisations in other features of this group (e.g. visual lateralisation in *Bombina variagata*: Bisazza *et al.*, 2002; and *Xenopus laevis*: Gouchie *et al.*, 2008). While it has been argued that the late stage of the *Bombina orientalis* tadpoles used by Goree & Wassersug (2001) may have contributed to the lack of any apparent bias (Malashichev & Wassersug, 2004), our *Bombina orientalis* were even further developed than those assessed previously. This could be explained by the transition in the direction of bias mentioned earlier, but it is unclear why these species would be distinctly affected. Regardless, the occurrence of lateralised behaviour in *Xenopus*, a common model organism for neurological and developmental studies and amenable to manipulation experiments on the direction of morphological lateralisations (Blackiston & Levin, 2013), means that there is considerable scope for greater understanding of lateralisation and its origin.

Disclosure statement

No potential conflict of interest.

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396 Table 1. The species used in the study, detailing the number of pairs used to collect the spawn for
397 the study, the number and location of spiracle(s), geographical region of origin, whether they are
398 considered Neobatrachians or a sister group.

Species	Parentage of spawn	Spiracle location[1]	Geographical region of origin[2]	Neobratrachia[3]
Rana temporaria	Single pair	Single, sinistral	Europe	Yes
Bufo bufo	Single pair	Single, sinistral	Europe	Yes
Xenopus laevis	Two pairs	Two symmetrical	sub-Saharan Africa	No
Xenopus borealis	Single pair	Two symmetrical	sub-Saharan Africa	No
Xenopus tropicalis[4]	Single pair	Two symmetrical	sub-Saharan Africa	No
Bombina orientalis	Two pairs	Single, virtually midline	central eastern Asia	No

399 [1] McDiarmid & Altig, 1999; [2] Frost, 2013; [3] Frost et al., 2006; [4] using *Xenopus tropicalis* as
400 according to Frost, 2013
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Table 2. The number of individuals from each of the six species that had either the right or left forelimb emerge first, whether they were tested for turning direction or not. Chi-squared values are presented (df = 1 for all tests; * $P < 0.01$; ^{NS} Not significant).

	Observation: Duration of forelimb asymmetry			Experiment: Assessed for turning direction		
	Right first	Left first	χ^2	Right first	Left first	χ^2
R. temporaria	3	14	**7.12***	13	12	0.04^{NS}
B. bufo	4	16	**7.20***	11	14	0.36^{NS}
X. laevis	12	8	0.80^{NS}	11	5	2.25^{NS}
X. borealis	23	26	0.18^{NS}	-	-	-
X. tropicalis	23	16	1.26^{NS}	-	-	-
B. orientalis	10	12	0.18^{NS}	20	10	3.33^{NS}

Table 3. Mean laterality Index (indicating right-ward turning bias) of all four species assessed for turning direction with neither, one and both forelimbs emerged. The t-statistic indicates whether the rightward-bias deviates from an index score of 0 (indicating no bias). Pooled G-value (df = 1) indicates whether the number of turns to the right for all individuals within a given category deviates from the expected 50:50 ration of right : left turns. The Heterogeneity G-value indicates whether individuals differ in their tendency to turn right or left, significant values indicate individuals differ in the strength or direction of bias. For Heterogeneity G-values the df for *Rana temporaria* and *Bufo bufo* are 24, for *Xenopus laevis* 16, and *Bombina orientalis* 29. Significant individual-level biases in turning direction occurred when all five turns were in a single direction (G = 6.93, p = 0.008). Only individuals with significant biases have been shown, and are displayed as the ratio of left turning individuals to right turning individuals. (^{NS} Not significant, * < 0.05, ** < 0.01. *** < 0.005, **** < 0.0001)

			R. temporaria	*B. bufo*	*X. laevis*	*B. orientalis*
Neither forelimb emerged	Laterality Index	Right-bias	0.36 ± 0.10	0.04 ± 0.09	0.35 ± 0.15	0.28 ± 0.07
		t-statistic	**3.49*** **	0.43^{NS}	**2.41***	**3.99****
	Repeated G-test	Pooled G-value	**16.57****	0.20^{NS}	**10.01*** **	**11.92*** **
		Heterogeneity G-value	**42.82***	30.37^{NS}	**35.49*** **	26.56^{NS}
	Individual-level bias (L:R ratio)		0 : 5	2 : 0	0 : 5	0 : 2
One forelimb emerged	Laterality Index	Right-bias	0.07 ± 0.11	0.17 ± 0.09	0.48 ± 0.13	0.23 ± 0.10
		t-statistic	0.67^{NS}	1.88^{NS}	**3.80*** **	**2.24***
	Repeated G-test	Pooled G-value	0.65^{NS}	3.55^{NS}	**18.80****	**7.77****
		Heterogeneity G-value	**41.83***	27.20^{NS}	**29.98***	**57.62***
	Individual-level bias (L:R ratio)		1 : 2	0 : 1	0 : 6	1 : 5
Both forelimbs emerged	Laterality Index	Right-bias	-0.14 ± 0.09	-0.04 ± 0.10	0.23 ± 0.08	0.01 ± 0.08
		t-statistic	-1.54^{NS}	-0.42^{NS}	**2.92***	0.17^{NS}

	Repeated G-test	Pooled G-value	2.32^{NS}	0.20^{NS}	**5.05***	0.03^{NS}
		Heterogeneity G-value	26.70^{NS}	30.541^{NS}	8.52^{NS}	31.55^{NS}
	Individual-level bias (L:R ratio)		1 : 0	1 : 0	0 : 0	1 : 1

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Figure 1: Percentage of individuals in a study that exhibited a left bias in turning direction at the mean Gosner stage from data published (●) in Wassersug & Yamashita (2002) and our data from stage 41 tadpoles (▲). Each point represents a species and stage class (R^2 = 24.3%, t = -3.15, p < 0.005).